

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
 US Department of Commerce
 United States Patent and Trademark
 Office, PCT
 2011 South Clark Place Room
 CP2/5C24
 Arlington, VA 22202
 ETATS-UNIS D'AMERIQUE
 in its capacity as elected Office

Date of mailing (day/month/year) 07 May 2001 (07.05.01)	Applicant's or agent's file reference 001978woMetg
International application No. PCT/EP00/07843	Priority date (day/month/year) 13 August 1999 (13.08.99)
International filing date (day/month/year) 11 August 2000 (11.08.00)	
Applicant BAR, Lily et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
 28 February 2001 (28.02.01)

☐ in a notice effecting later election filed with the International Bureau on:

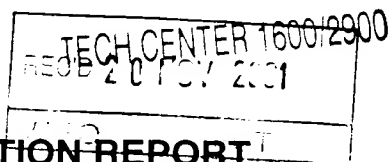
2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Nestor Santesso
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PCT



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

9/744973



14

Applicant's or agent's file reference 001978woMe	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP00/07843	International filing date (day/month/year) 11/08/2000	Priority date (day/month/year) 13/08/1999
International Patent Classification (IPC) or national classification and IPC A61L24/10		
Applicant OMRIX BIOPHARMACEUTICALS S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 4 sheets, including this cover sheet.
- ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).
- These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 28/02/2001	Date of completion of this report 15.11.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Cattell, James Telephone No. +49 89 2399 8468 

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/07843

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

Description, pages:

1-16 as originally filed

Claims, No.:

1-8 as originally filed

Drawings, sheets:

1/5-5/5 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP00/07843

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims 1-8
	No: Claims
Inventive step (IS)	Yes: Claims 1-8
	No: Claims
Industrial applicability (IA)	Yes: Claims 1-8
	No: Claims

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP00/07843

V.

- 1). Document D1 (US-A-4 960 757) discloses in claim 11 a process for the preparation of fibrinogen involving sucrose, glycine and heating to 60° for 10 to 24 hours. D1, col. 3, lines 1 to 3 describes the removal of fibrin polymers. The use of fibrinogen multimers, as claimed, could not be derived from this document. The claims therefore appear to meet the requirements of Article 33 PCT.

VIII.

- 3). For the assessment of the present claims 1 to 4 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

PCT REQUEST

Original (for SUBMISSION) - printed on 11.08.2000 02:57:39 PM

0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form - PCT/RO/101 PCT Request Prepared using	PCT-EASY Version 2.91 (updated 01.07.2000)
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	European Patent Office (EPO) (RO/EP)
0-7	Applicant's or agent's file reference	001978woMetg
I	Title of invention	FIBRINOGEN MULTIMERS
II	Applicant	
II-1	This person is:	applicant only
II-2	Applicant for	all designated States except US
II-4	Name	OMRIX BIOPHARMACEUTICALS SA
II-5	Address:	200 Chaussée de Waterloo B-1640 Rhode-St-Genése Belgium
II-6	State of nationality	BE
II-7	State of residence	BE
III-1	Applicant and/or inventor	
III-1-1	This person is:	applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	BAR,, Lily
III-1-5	Address:	Weitzmann Science Park P.O. Box 619 76106 Rehovot Israel
III-1-6	State of nationality	IL
III-1-7	State of residence	IL

PCT REQUEST

Original (for SUBMISSION) - printed on 11.08.2000 02:57:39 PM

III-2	Applicant and/or inventor	applicant and inventor US only NUR,, Israel Weitzmann Science Park P.O. Box 619 76106 Rehovot Israel IL IL
III-2-1	This person is:	
III-2-2	Applicant for	
III-2-4	Name (LAST, First)	
III-2-5	Address:	
III-2-6	State of nationality	
III-2-7	State of residence	
IV-1	Agent or common representative; or address for correspondence The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	agent MEYERS,, Hans-Wilhelm P.O. Box 10 22 41 D-50462 Köln Germany 0221-91 65 20 0221-13 42 97 mail@dompatent.de
IV-1-1	Name (LAST, First)	
IV-1-2	Address:	
IV-1-3	Telephone No.	
IV-1-4	Facsimile No.	
IV-1-5	e-mail	
IV-2	Additional agent(s)	
IV-2-1	Name(s)	
V	Designation of States	AP: GH GM KE LS MW MZ SD SL SZ TZ UG ZW and any other State which is a Contracting State of the Harare Protocol and of the PCT EA: AM AZ BY KG KZ MD RU TJ TM and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT EP: AT BE CH&LI CY DE DK ES FI FR GB GR IE IT LU MC NL PT SE and any other State which is a Contracting State of the European Patent Convention and of the PCT OA: BF BJ CF CG CI CM GA GN GW ML MR NE SN TD TG and any other State which is a member State of OAPI and a Contracting State of the PCT
V-1	Regional Patent (other kinds of protection or treatment, if any, are specified between parentheses after the designation(s) concerned)	

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 001978woMetg	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 00/ 07843	International filing date (day/month/year) 11/08/2000	(Earliest) Priority Date (day/month/year) 13/08/1999
Applicant OMRIX BIOPHARMACEUTICALS SA		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 2 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☒ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

1
☐ None of the figures.

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61L24/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 960 757 A (KUMPE GERHARDT ET AL) 2 October 1990 (1990-10-02) the whole document ---	1-8
A	EP 0 856 317 A (OMRIX BIOPHARM SA) 5 August 1998 (1998-08-05) page 2, line 22 - line 40 ---	5-8
A	US 4 650 678 A (FUHGE PETER ET AL) 17 March 1987 (1987-03-17) the whole document ---	1-8
A	US 4 362 567 A (SCHWARZ OTTO ET AL) 7 December 1982 (1982-12-07) the whole document -----	1-8

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

18 December 2000

Date of mailing of the international search report

28/12/2000

Name and mailing address of the ISA

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 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
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Authorized officer

Heck, G

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/07843

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4960757	A	02-10-1990	DE 3230849 A	23-02-1984
			AT 47525 T	15-11-1989
			AU 587365 B	17-08-1989
			AU 1810983 A	23-02-1984
			CA 1203166 A	15-04-1986
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			GR 78934 A	02-10-1984
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			NO 832977 A,B,	20-02-1984
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			PT 77213 A,B	01-09-1983
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			AU 724715 B	28-09-2000
			AU 6214598 A	25-08-1998
			BR 9807043 A	28-03-2000
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			AR 231233 A	31-10-1984
			AT 22806 T	15-11-1986
			AU 556068 B	23-10-1986
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			ES 519469 D	16-03-1984
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			FI 830361 A,B,	05-08-1983
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			JP 2025664 C	26-02-1996
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			ZA 8300726 A	26-10-1983
			AT 359652 B	25-11-1980
			AT 118979 A	15-04-1980
			BE 881466 A	16-05-1980
			CA 1128859 A	03-08-1982

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/07843

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4362567 A		CH 649711 A	14-06-1985
		DE 3002934 A	28-08-1980
		DK 65580 A, B	16-08-1980
		ES 488430 D	16-12-1980
		ES 8101381 A	16-03-1981
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		GB 2041942 A, B	17-09-1980
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		JP 55110556 A	26-08-1980
		JP 63040546 B	11-08-1988
		NL 8000935 A, B	19-08-1980
		SE 453799 B	07-03-1988
		SE 8000469 A	16-08-1980
		SE 462613 B	30-07-1990
		US 4414976 A	15-11-1983

Use of Fibrinogen Multimers

The invention is concerned with the use of fibrinogen multimers as an ingredient of a fibrin sealant and a method of producing a fibrin sealant comprising multimers.

Fibrin sealant is a generic name covering a complex of plasma derivatives which are usually composed of proteins, which mimic those active in the last stage of the coagulation cascade, from the activation of soluble fibrinogen by thrombin to the formation of insoluble protein fibrin (Matras 1985, Sierra 1993, Spotnitz et al 1987 and Spotnitz 1995). Most of the commercial fibrin sealants are composed of two major components - thrombin supplemented with Ca^{++} ions and fibrinogen supplemented with varying amounts of factor XIII. Once both components are mixed, the thrombin splits off fibrinopeptides A and B from the respective Aa and Bb chains of fibrinogen to form fibrin monomers that are polymerized by hydrogen bonding to form a soluble fibrin clot. Factor XIII, activated by thrombin in the presence of Ca^{++} ions, catalyzes crosslinking between the fibrin molecules, increasing the mechanical strength of the clot and reducing its susceptibility to proteolytic cleavage (Gladner and Nossal 1983).

Most fibrin sealants are prepared from either plasma cryoprecipitate or from Cohn fraction I, as sources of fibrinogen. Cryoprecipitate is obtained by thawing frozen plasma at 2-4°C, resulting in a paste that is recovered by centrifugation. Cryoprecipitate contains fibrinogen, fibronectin, vonWillebrand factor and other proteins that are co-precipitated with the above proteins, such as albumin, IgG, IgM, IgA, vitamin K-dependent-clotting factors and factor XIII (FXIII). Since the use of plasma cryoprecipitate, as a source for the sealant, may result in a lower activity of factor FXIII (Radosevich et al, 1997) some of the companies add exogenous factor XIII from various sources. Products manufactured from cryoprecipitate such as TissuCol (which has recently received a free marketing authorization in the US) and BeriPlast have

CONFIRMATION COPY

been in the European market for more than a decade, however FXIII is not added to any of these products in the US. Cohn fraction I, or equivalent products, are prepared by ethanol precipitation of plasma by 8-10% ethanol at -30°C - -50°C at neutral pH. BioCol™ produced by Plasma Product services, Lille France is such product. The product is manufactured by processing the whole plasma. Prior to a 10% cold ethanol precipitation, the plasma is thawed at 37°C for at least 6 hours followed by recovery of the fibrinogen by centrifugation. The paste is then reconstituted in Tris/lysine buffer and subjected to a second 10% cold ethanol precipitation for an additional 10-12 hours. This allows for highly purified fibrinogen, which is then treated by solvent detergent for virus inactivation. The final product is lyophilized for prolonged storage at 4°C (Burnouf-Radosevich et al 1990).

Fibrinogen integrity and structure are of most importance for the performance of fibrin sealant. The rates of polymerization, binding to the fibrin receptor and susceptibility to fibrinolytic digestion have a critical influence on the performance in the clinical arena.

Fibrinogen, a tri-domainal (two D domains and a central E domain) disulfide bond molecule about 47 nm in length, is composed of two symmetrical half-molecules, each consisting of a set of three different polypeptide chains termed A α , B β and γ . The halves are covalently joined in the central amino-terminal E domain by five disulfide bridges. Reciprocal disulfide bridges are formed between the $\gamma 8$ and $\gamma 9$ position, thus orienting the γ chains in an anti-parallel direction (Mosesson, 1997). Removal of the fibrinopeptides A and B upon activation by thrombin induces a spontaneous polymerization of the fibrin monomer molecule via noncovalent binding.

Although fibrinogen has insufficient centers for such polymerization, nevertheless, in suitable conditions and without any enzymatic action, such as at high fibrinogen concentration, low ionic strength, or long incubation at 4°C , fibrinogen is polymerized (Rozenfeld and Vasileva 1991). Fibrinogen is liable to

undergo self-association according to some authors to form structures that are either similar to fibrin (Cohen et al 1966; Gollwitzer et al 1983; Ugarova et al 1987) or to quasi-globular particles (Becker 1987).

Rozenfeld and Vasileva (1991) found that the molecules of fibrinogen undergo a spontaneous modification of their carboxyl terminals and bind end to end into flexible polymer chains. On attaining a critical length, the single-filament polymers twist into a coil and aggregate to form branch molecules in which the segments are packed sufficiently densely to resemble strong hydrated globular particles. The formation, under the influence of factor XIIIa, of ϵ/γ -glutamyl - lysine covalent bonds produced only insignificant changes in the spatial organization of the fibrinogen aggregates. Covalent dimerization of the γ -chains restricts the structural flexibility of polymers, but linking of the α -chains provides progressive compaction of the structure with an increase in its molecular weight. Electrophoresis of reconstituted samples shows that the coil-shaped chains of fibrinogen oligomers prevent the complete enzymatic linking of the γ -chains. From the results of their work, the authors suggest that the accelerated assembly of multimolecular aggregates, seen in the presence of Factor XIIIa, may be explained by the stabilization of intermediate complexes of fibrinogen, which make the spontaneous transition from a stable native state to the active state irreversibly. However a more recent paper by Mosesson claims that fibrinogen molecules become covalently crosslinked by factor XIIIa in the same sequence as in fibrin, i.e. first the α and then the γ chain (Mega et al 1988, and Mosesson et al 1995).

It is an object of the present invention to provide a fibrin sealant having improved properties.

According to the invention fibrinogen multimers having at least 6 fibrinogen units are used as an ingredient of a fibrin sealant. Preferably, 6-15 fibrinogen units in the multimer are used. Still more preferred are 6-9 fibrinogen units. In particular 8 or 9 units are present in the multimer.

A method for manufacturing the fibrin sealant having fibrinogen multimers with at least 6 units comprises the steps of

- resuspending cryoprecipitate
- adding sucrose to yield a concentration of 60 to 70% (w/w),
- adding glycine to yield a concentration of 0.1 to 0.3 M,
- heating to 60°C for 15 - 20 hours,
- removing the glycine and the sucrose by dialysis,
- adding a protease inhibitor.

Preferably, the dialysis is conducted against a buffer comprising NaCl, glycine and CaCl_2 .

Typically, the protease inhibitor is tranexamic acid in a concentration of 8 - 12% (w/w) and/or arginine in a concentration of 1 - 3% (w/w).

Subject of the present invention is also a fibrin sealant comprising multimeric fibrinogen having at least 6 fibrinogen units.

In order to investigate the influence of the nature of the fibrinogen component in fibrin sealants, the immuno-reaction of the non-reduced agarose SDS electrophoresis method was modified first established by Connaghan et al (1985) and by Proietti et al (1990). The modification of this semi-quantitative method initially designed to monitor fibrin crosslinked polymers and its degradation products in plasma, was used for monitoring the number of fibrinogen monomers/units and their amounts in each oligomer in various fibrinogen preparations and in fibrin sealants.

Even though a detailed protocol for fibrinogen identification using agarose horizontal gel electrophoresis has been published in 1990, the method was unsatisfactory for the separation of high molecular wt. fibrinogen protein

(Proietti et al 1990). The method developed by Reines (1990) for the detection of vWF multimers to be used for the immunodetection of fibrinogen, was modified as follows: The Horizontal Electrophoresis was performed using the Reines et al (1990) detailed protocol for vWF multimers, however, the detection of fibrinogen multimers and the analysis method were of Proietti et al (1990), with some modifications (Proietti et al 1990). Briefly, samples were prepared by diluting to a protein concentration of 0.1mg/ml in a solution of 70 mM Tris, 4 mM EDTA, 2.4 % SDS, 9 M urea and 0.1 g/L bromophenol blue added as a marker and incubated at 60°C for 30 min. Agarose was dissolved to a final concentration of 1 % (stacking gel), 1.6 % (cathode gel) and 2 % (separating gel) by boiling in 70 mM Tris, 4 mM EDTA and 4 % SDS (stacking gel buffer) or 100 mM Tris, 150 mM glycine and 0.1 % SDS (cathode gel buffer and cathode buffer) or 200 mM Tris, 100 mM glycine and 0.4 % SDS (separating gel buffer). Gels (cathode, stacking and separating gels) were cast at >50°C and allowed to polymerize for at least 2 hours for each gel. Samples were loaded at 0.6-0.9 µl per well, then electrophoresis was performed toward the anode at 600 - 650 volts/running hours at a constant temperature of 16°C on a flat bed electrophoresis apparatus (Pharmacia Fine Chemicals, Sweden). The running buffer was connected to the gel via 4-8 layers of filter paper immersed in electrode buffer (the anode buffer had half the strength of the cathode buffer). Electrophoresis proceeded until the tracking dye had migrated a distance of 6-8 cm. After electrophoresis, the gel was removed, the stacking and cathode gels taken away and the separating gel washed in transfer buffer (25 mM Tris-HCl, 192 mM glycine pH 8.3 buffer, diluted 1:2 in 20 % methanol) for approximately 1 minute.

Immunoblotting was performed onto a Nitrocellulose membrane (Optiran BA-S83 S&S Germany), cut to size, using the capillary blotting procedure (Reines et al 1990). After protein transfer, the nitrocellulose membrane was carefully removed from the capillary blotting unit and the residual protein binding capacity blocked by immersion in blocking buffer (0.5% I-Block Tropix, USA. 0.05% Sodium Azide in PBS) for 1 hour. Immunodetection of fibrinogen

multimers was done using a goat polyclonal anti-human fibrinogen antibody (Sigma, USA), and a rabbit polyclonal anti-goat immunoglobulin alkaline phosphatase conjugate (Sigma, Mo USA) was used for immunostaining.

SDS-Polyacrylamide electrophoresis was done in a vertical electrophoretic unit (Hoefer Scientific Instruments, San Francisco, CA USA) using a reducing system containing 250 mM Tris, 40 % glycerol, 4% SDS and 4 % β -Mercaptoethanol (loading buffer). The separating gel was prepared as a gradient from 6 to 12 % acrylamide concentration (from a 30 % acrylamide and 0.8 % bisacrylamide stock solution) containing 10 % ammonium persulfate and 0.02 % TEMED. The samples were mixed with 4 x loading buffer to $\frac{1}{4}$ of the final volume and heated in a dry block at 95°C for 5 minutes. The gel was run at 200V and the electrophoresis was stopped when the Bromophenol Blue reached the bottom of the gel. Immunodetection was performed using a semi-dry immuno-blotter (Hoefer Scientific Instruments, San Francisco, CA USA) onto a nitrocellulose membrane using the same antibodies and protocol as for the horizontal electrophoresis.

Figure 1 shows the immunodetection of fibrinogen multimers of three plasma fractionations in-process sources of fibrinogen. One source derived from Paste I was supplemented with Factor FXIIIa. (From right to left) Lanes 1-6: cryoprecipitate; Lanes 7-12: purified fibrinogen; Lanes 13-18: purified Fibrinogen + FXIIIa; Lanes 19-21: solvent detergent paste I; Samples were taken during the process of the invention: Lanes 1, 7, 13 and 19: samples taken at the beginning of the process. Lanes 2, 8, 14 and 20: samples taken after the addition of sucrose and glycine. Lanes 3, 4, 9, 10, 15, 16 and 21: taken after incubation at 60°C for 17 hours. Lanes 5, 11, and 17: taken after dialysis and ultrafiltration. Lanes 6, 12, 18, and 22: taken after the formulation with 10% Tranexamic acid and 2% Arginine.

All the samples were loaded at 0.7-0.9 μ g per lane.

Figure 2 shows the imunodetection of fibrinogen multimers electrophoresed on agarose SDS gel. Fibrinogen samples are of eight batches of the product of the present invention - lane 4: #C31102; lane 5: C30092; lane 6: C29081; lane 7: C27071; lane 8: C25061; lane 9: C22052; lane 10: 14032; lane 11: C12022. Four additional commercial sources of fibrinogen were added - lane 1: Tisseel™; lane 2: BeriPlast; lane 3: BioCol and lane 12: fibrinogen from Enzyme Research Inc. All the samples were loaded at 0.65µg per lane.

Figure 3 shows the immunodetection of fibrinogen multimers of three commercial preparations of fibrin sealants: From left to right. Lane 12: Tisseel™; lane 11: BioCol; lane 10: Fibrinogen from Enzyme research Inc.; lane 9: product prepared according to the method of the invention. Other lanes are fibrinogen production in process samples taken after the following steps: lane 1: cryoprecipitate resuspension sample-BC01; lane 2: aluminum alhydrogel absorption sample-BC02; lane 3: SD treatment -BC04; lane 4: SD removal-BC06; lane 5: after pasteurization-BC09; lane 6: addition of stabilizers - BC10; lane 7: final concentration-BC11, lane 8: sterile bulk-BC12. All the samples were loaded at 0.65µg per lane.

Figure 4 shows a western blot-immunodetection of various formulations of urea non-reduced fibrinogen multimers of product prepared according to the method of the invention, run by Agarose-SDS gel electrophoresis. Lane 1: no Arginine and no Tranexamic acid; lane 2: 2% Arginine; lane 3: 2% Arginine and 5% Tranexamic acid; lane 4: 2% Arginine and 10% Tranexamic acid; lane 5: 10% Tranexamic acid, lane 6: 1% Arginine and 10% Tranexamic acid; lane 7: 4% Arginine and 10% Tranexamic acid, lane 8: Fibrinogen from Enzyme Research Inc.; lane 9: Tisseel™; lane 10: BioCol. All samples were loaded at 0.65 µg protein per lane.

Figure 5 shows western blot-immunodetection of various formulations of reduced fibrinogen multimers of product prepared according to the method of the invention, migrated on PAGE-SDS gel electrophoresis. Lane 1: No Arginine

and no Tranexamic acid; lane 2: 2% Arginine; lane 3: 2% Arginine and 5% Tranexamic acid; lane 4: 2% Arginine and 10% Tranexamic acid; lane 5: 10% Tranexamic acid; lane 6: 1% Arginine and 10% Tranexamic acid; lane 7: 4% Arginine and 10% Tranexamic acid, lane 8: Fibrinogen from Enzyme Research Inc.; lane 9: Tisseel™. All samples were loaded at 10 µg protein per lane.

The initial fibrinogen multimeric state in various fibrinogen sources and the effect of a production process on the polymerization was identified. Three fibrinogen sources were used: 1) Re-suspension of cryoprecipitate (Sample A, Omrix Biopharmaceuticals LTD (Israel); 2) purified fibrinogen, derived from Cohn Fraction I (Sample B, Enzyme Research, USA) or purified fibrinogen supplemented with FXIIIa (Sample C, Enzyme Research USA) and 3) solvent detergent treated fraction I (Sample D, from OctaPharma (Austria)). They were treated with the process of the present invention. Pasteurization, dialysis and formulation with Tranexamic acid and Arginine and at each stage of the procedure, samples have been taken to test the state of polymerization (see Table 1).

Samples labeled A1, B1, C1 and D1 are re-suspended fibrinogen samples that have been directly taken for testing (lanes 1,7,13 and 19 in figure 1).

Samples labeled A2, B2, C2 and D2 are re-suspended fibrinogen samples mixed with 65% (w/w) sucrose and 0.2 M glycine, incubated at 37°C for less than 4 hours, heated to 60°C to allow for complete sucrose dissolution (lanes 2,8,14 and 20 in figure 1).

Samples labeled A3, B3, C3 and D3 are re-suspended fibrinogen samples mixed with sucrose and glycine, incubated at 37°C for less than 4 hours and incubated at 60°C for 17 hours (lanes 3,9,15 and 21 in figure 1).

Samples labeled A4, B4 and C4 are re-suspended fibrinogen samples mixed with sucrose and glycine, incubated at 37°C for less than 4 hours. Then,

incubation at 60°C for 17 hours was performed and dialyzed against 10 mM citrate buffer 120 mM NaCl, 120 mM glycine and 1mM CaCl₂ (lanes 4,10 and 16 in figure 1).

Samples labeled A5, B5, C5 and D5 are re-suspended fibrinogen samples mixed with sucrose and glycine, incubated at 37°C for less than 4 hours, incubated at 60°C for 17 hours, and dialyzed against 10 mM citrate buffer 120 mM NaCl, 120 mM glycine and 1mM CaCl₂. The resulting solution was then formulated with 10% Tranexamic acid and 2% Arginine (lanes 5,11 and 17 in figure 1). For additional clarifications see Table 1.

Table 1: Experimental design of fibrinogen treatments, sources, addition of Factor XIIIa, incubation at high temperature, formulation during the pasteurization with sucrose and glycine and addition of final product stabilizers, Tranexamic acid and Arginine. The effect of all these parameters on the fibrinogen multimeric state was tested.

Lane #	No. of sample	Name of sample	Procedure - end point of treatment	Incubation temperature	Incubation time at the last point
1	A1	Cryoprecipitate	-	-	0
2	A2	Cryoprecipitate	Sucrose, glycine	37°C	4h
3	A3	Cryoprecipitate	pasteurization	60°C	17h
4	A3'				
5	A4	Cryoprecipitate	dialysis ultrafiltration	and RT	3h+2.5h
6	A5	Cryoprecipitate	add 10%TEA, Arginine	2% RT	1h
7	B1	Purified Fibrinogen		RT	0
8	B2	Purified Fibrinogen	Sucrose, glycine	37°C	2h
9	B3	Purified Fibrinogen	pasteurization	60°C	17h
10	B3'				
11	B4	Purified Fibrinogen	dialysis ultrafiltration	and RT	3h+2.5h
12	B5	Purified Fibrinogen	add 10%TEA, Arginine	2% RT	1h
13	C1	Purified Fibrinogen +FXIII		RT	0
14	C2	Purified Fibrinogen +FXIII	Sucrose, glycine	37°C	2h
15	C3	Purified Fibrinogen +FXIII	pasteurization	60°C	17h
16	C3'				
17	C4	Purified Fibrinogen +FXIII	dialysis ultrafiltration	and RT	3h+2.5h
18	C5	Purified Fibrinogen FXIII	add 10%TEA, Arginine	2% RT	1h
19	D1	Fraction I S/D		RT	0
20	D2	Fraction I S/D	Sucrose, glycine	37°C	1.5h
21	D3	Fraction I S/D	pasteurization	60°C	17h

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Figure 1 shows that the cryo-precipitate fibrinogen source has more fibrinogen multimers than the other fibrinogen sources, as it can be seen in bands 1, 7, 13 and 19. On the other hand, the 60°C incubation increased the number and the amounts of fibrinogen units per multimer and the percentage of multimeric fibrinogen. However, this effect is most evident in purified fibrinogen and in Fraction I (lanes 9 and 15). The effect of FXIIIa addition is marginal, probably since all fibrinogen sources from plasma contain traces of FXIII.

The effect of fibrinogen source is further demonstrated in Figure 2. Figure 2 shows multimers of fibrinogen of fibrin sealant produced using the process of the invention, from cryoprecipitate after double virus inactivation (solvent detergent and pasteurization) and stabilizer addition from eight batches. Figure 2 also presents the Multimeric State of four fibrinogen concentrates from commercial sources (Purified fibrinogen Enzyme Research, TissuCol, Biocol and Beriplast). As can be noted from the figure, purified fibrinogen (Enzyme research Inc.) derived from Fraction I is primarily a monomer. However that preparation contains a small amount of fibrinogen dimeric structure. On the other hand, commercial fibrinogen products, which compose part of fibrin sealants, contain fibrinogen multimers up to pentamers while the fibrin sealant of the present invention has high multimeric fibrinogen structures reaching up to octamers or nonamers. Furthermore, the batch to batch reproducibility, relative to the high multimeric state of fibrinogen, is evident.

The first part of the study revealed that pasteurization has a major effect on the polymerization of fibrinogen from Fraction I. However, the effect on cryoprecipitate was not fully demonstrated. In the second part of the study, the changes in the Multimeric State of fibrinogen during the production process and the batch to batch variability were examined. Samples were withdrawn from the process and the material was subjected to examination by immunodetection after agarose gel electrophoresis. Figure 3 shows fibrinogen immunodetection of samples obtained following each production step: cryo-resuspension - lane 1; alhydrogel absorption lane 2; SD treatment - lane 3,

SD removal - lane 4, post-pasteurization - lane 5, stabilizer addition - lane 6, final concentration - lane 7, sterile bulk - lane 8 and final container-lane 9, purified fibrinogen-lane 10 and the two commercial fibrinogen concentrates (Tissel and Biocol)-lanes 11 and 12 respectively. The figure clearly shows that even though the cryo-resuspension contains six-mers (lane 1), the addition of stabilizers (lane 6) promoted fibrinogen polymer formation since lane 6, sample after stabilizers addition, contains at least one more multimeric unit than the previous sample, after pasteurization (lane 5). This clearly indicates that the addition of Arginine and Tranexamic acid had an effect on the fibrinogen multimeric state.

In order to examine the effect of each of the stabilizer, a sample was withdrawn from the process prior to stabilizer addition. At this point, several combinations of Arginine and Tranexamic acid were formulated containing between 0 - 4 % and 0 - 10 % (w/w) respectively. The different formulations were frozen, stored for seven days and thawed at 37°C. After thawing, the samples, together with purified fibrinogen and commercial fibrin concentrates, were run on a horizontal SDS-Agarose non reduced and vertical reduced β -mercaptoethanol SDS-PAGE) electrophoresis. The results are shown in figures 4 (horizontal SDS-Agarose non-reduced) and 5 (SDS-PAGE reduced β -mercaptoethanol SDS-PAGE). The horizontal agarose gel electrophoresis followed by specific immunodetection of fibrinogen shows that addition of Arginine promotes multimer formation but the main contribution to its effect is done by the addition of Tranexamic acid. This finding is supported by samples that contains 0% Arginine and 10 % TEA which produced more and larger fibrinogen multimers than 2 % Arginine and 0% TEA. The same phenomenon can be seen on the reduced β -mercaptoethanol SDS-PAGE conducted on the same samples (Figure 5). A new band with a molecular weight above 250 kD appears in all formulations but not in the formulation with no stabilizers and not in the commercial concentrated fibrinogen tested (fibrinogen from Enzyme Research Inc and Tisseel™). Furthermore, in the samples formulated with 10% Tranexamic acid, this high molecular weight band is much more intense than

other formulations, indicating an increase in the ratio of a high number of multimeric units. Another observation that can be made from the figure is the absence of almost all the γ - γ and α - γ fibrinogen dimers from samples produced according to the method of the invention that are quite abundant in other fibrinogen concentrates (Siebenlist and Mossesson, 1996). This suggests that the increase of fibrinogen polymers is at the expense of fibrinogen dimers. Moreover, the addition of Tranexamic acid and Arginine might stabilize the fibrinogen polymers or even drive the reaction towards a higher multimeric state.

It was established that native fibrinogen, left undisturbed, forms reversible temperature- dependent aggregates (Becker and Waugh, 1980; Becker, 1987). Based on light scattering and viscosity examinations Rozemfeld and Vasileva, (1991) suggested that fibrinogen undergoes spontaneous self-association through its D domain to form flexible end to end polymer chains. They found that self-association accrued more rapidly when factor XIIIa was present, but the spatial arrangement of the fibrinogen molecules remained the same. Masesson and Siebenlist 1995 supply direct ultra-structural evidence for such associations (Siebenlist and Mossesson, 1996). However, the extent of these polymers has never been investigated in highly concentrated fibrinogen of the commercial available fibrin sealants. Furthermore in this manuscript, for the first time, evidence is been given for a fractionation production process which might lead to such high multimeric appearance of fibrinogen.

As noted in figure 1 and 3, Cryoprecipitate production resulted in a high multimeric structure. However, during the production process, some of the highest molecular multimers (seven to nine fibrinogen units) are lost, probably due to filtration and precipitation. Later steps in the process of the invention, such as pasteurization and stabilization with negatively charged amino acid, recovered the high multimeric state and stabilized it even during a prolonged storage (see figure 2). Other commercial product such as BeriPlast™ and Tisseel™ also derived from cryoprecipitate, however, the final product contains

lower multimeric fibrinogen. Both contain 4-5 fibrinogen units whereas the product prepared according to the process of the invention contains about 8-9 fibrinogen units. A different manufacturing process and different formulation may explain this difference. Both are formulated with Aprotinin and a low concentration of amino acid whereas the fibrin sealant prepared according to the process of the invention is formulated with a high concentration of Tranexamic acid and Arginine. This high salt concentration probably facilitated the formation of the high molecular wt. multimers. BloCol on the other hand is produced from a modification of Cohn fraction I. This starting material has a low multimeric state (see figure 1), probably because of the presence of a high concentration of ethanol which suppresses the enzymatic activity of FXIII during the precipitation of fibrinogen.

The clinical relevance of the multimeric state has to be assessed carefully by conducting well designed animal experiments. Since such experiments involve strong commercial interest, these experiments have to be performed in a well-controlled manner. The animal model consists of spraying of adhesive/fibrin sealant to control bleeding caused by partial liver resection in heparinized rabbit. Results show that the bleeding time and the amount of glue used were significantly shorter and lower for the fibrin sealant prepared according to the process of the present invention than in other fibrinogen concentrate tested (BeriPlast™ and Tisseel™). These outcomes may indicate that it has better adhesive properties to tissue than other glues. There are also reports indicating that it seems to work also at low temperature in indications such as liver transplantation. One explanation of the high adherence to tissue may be related to the high multimeric state of it as compared to the other fibrin sealants tested. These large molecules, some of them of more than 2×10^6 D molecular wt, are structurally very similar to the fibrin network. These protein macromolecules have better adhesive forces than their monomeric units, very similar to the phenomenon known in vWF Multimers.

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Claims

1. Use of fibrinogen multimers having at least 6 fibrinogen units as an ingredient of a fibrin sealant.
2. Use according to claim 1 having 6 - 15 fibrinogen units as an ingredient of a fibrin sealant.
3. Use according to claim 1 wherein the fibrinogen multimers have 6 to 9 fibrinogen units.
4. Use according to any of claims 1 to 3 wherein the amount of fibrinogen multimers having 6 to 9 fibrinogen units is at least 5% (w/w) of total fibrinogen.
5. A method for the production of a fibrin sealant comprising fibrinogen multimers having at least 6 fibrinogen units comprising the steps of
 - resuspending cryoprecipitate
 - adding sucrose to yield a concentration of 60 to 70% (w/w),
 - adding glycine to yield a concentration of 0.1 to 0.3 M,
 - heating to 60°C for 15 - 20 hours,
 - removing the glycine and the sucrose by dialysis,
 - adding a protease inhibitor.
6. The method of claim 5, wherein the dialysis is conducted against a buffer comprising NaCl, glycine and CaCl₂.

7. The method of claim 5, wherein the protease inhibitor is tranexamic acid in a concentration of 8 - 12% (w/w) and/or arginine in a concentration of 1 - 3% (w/w).
8. A fibrin sealant comprising multimeric fibrinogen of at least 6 fibrinogen units.

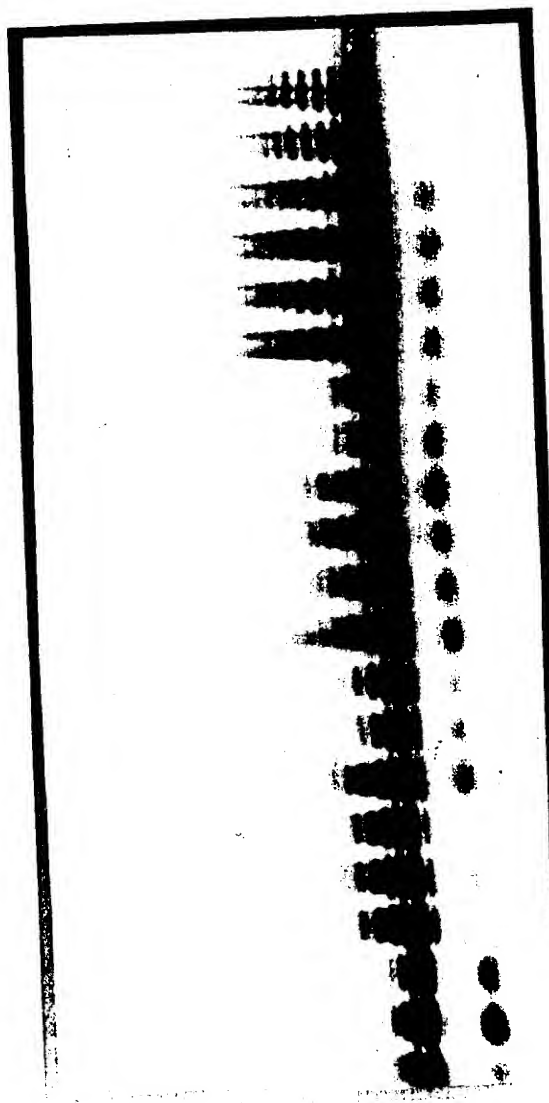


Fig. 1

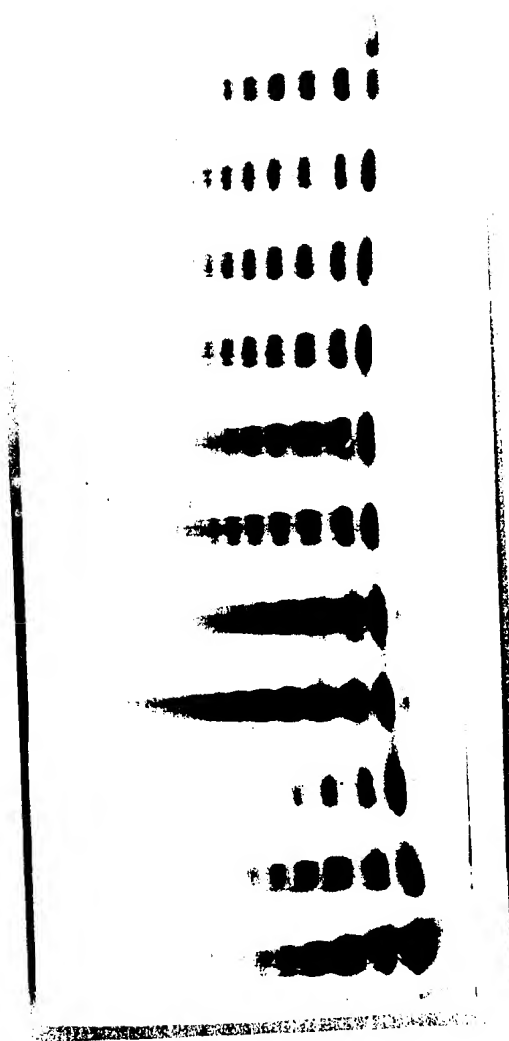


Fig. 2

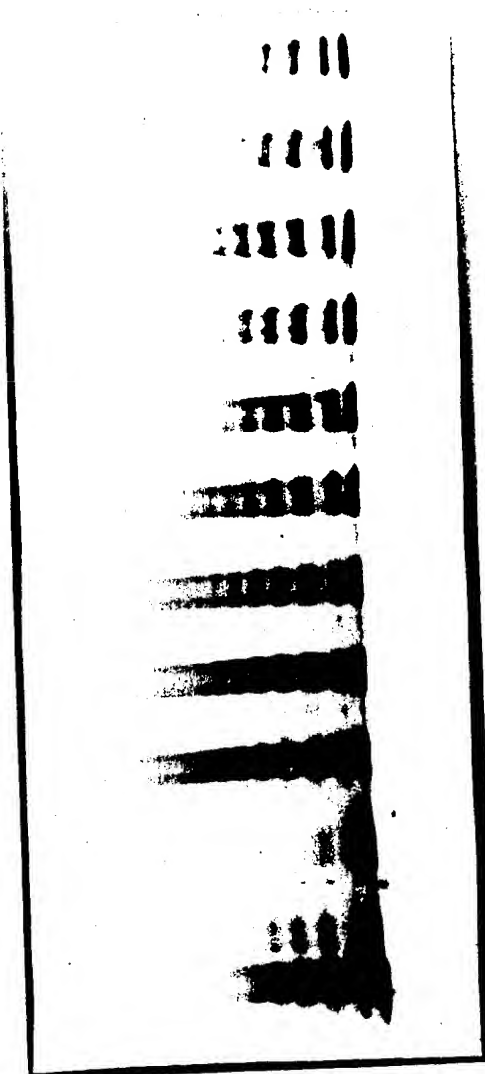


Fig. 3

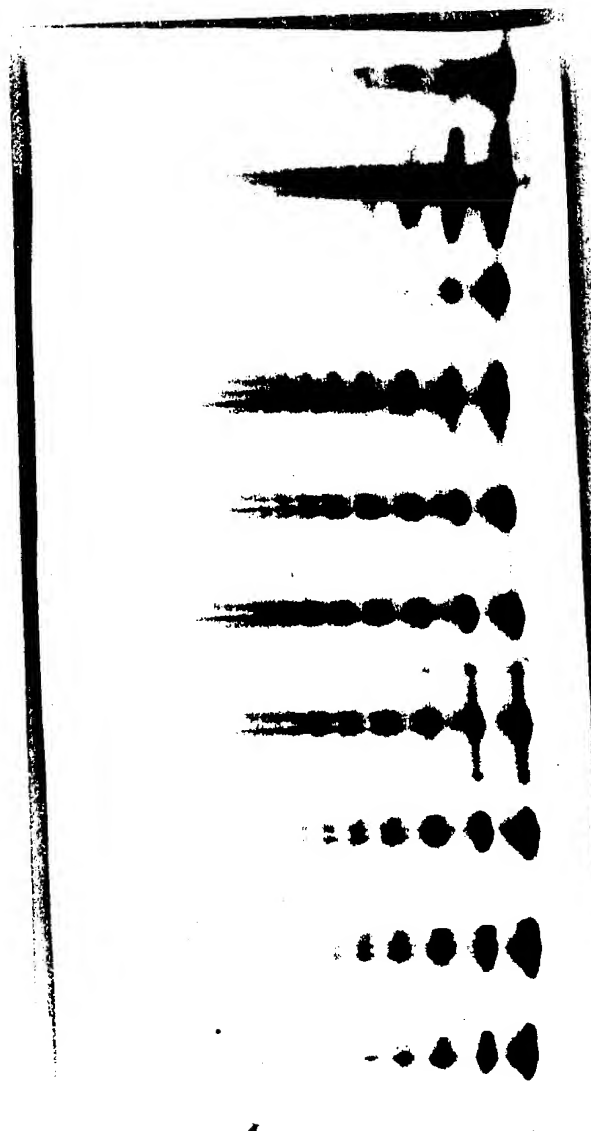


Fig. 4

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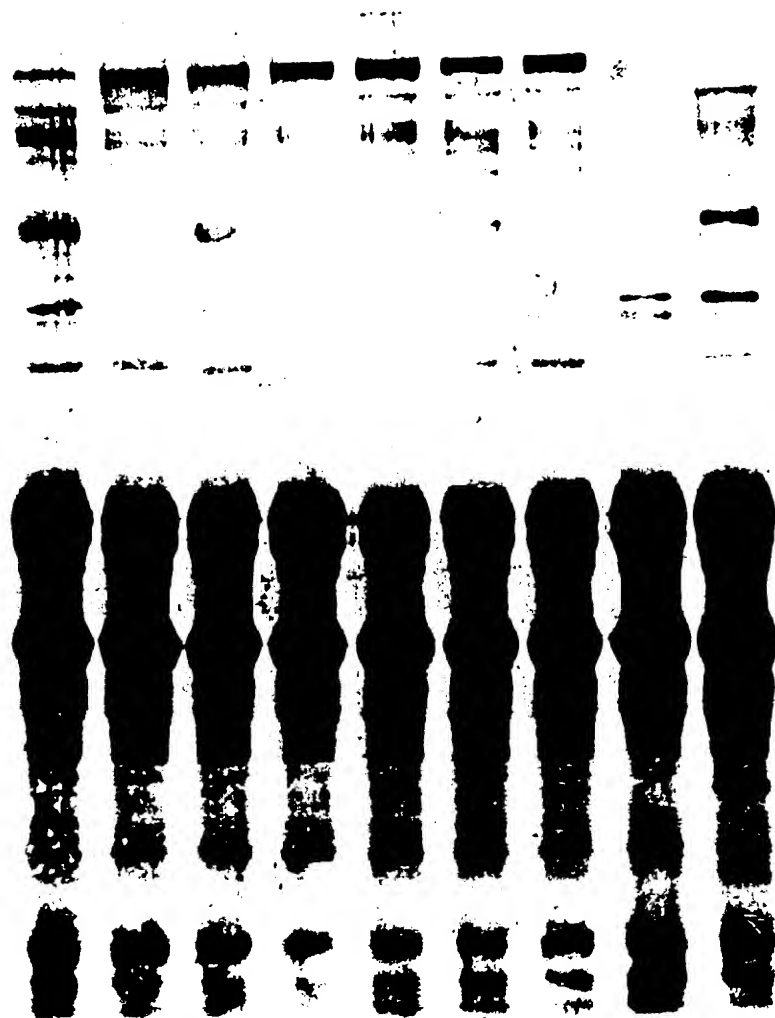


Fig. 5

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